

The High-Pathogenicity Island of *Yersinia enterocolitica* Ye8081 Undergoes Low-Frequency Deletion but Not Precise Excision, Suggesting Recent Stabilization in the Genome

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Highly pathogenic strains of *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are characterized by the possession of a pathogenicity island designated the high-pathogenicity island (HPI). This 35- to 45-kb island carries an iron uptake system named the yersiniabactin locus. While the HPIs of *Y. pestis* and *Y. pseudotuberculosis* are subject to high-frequency spontaneous deletion from the chromosome, we were initially unable to obtain HPI-deleted *Y. enterocolitica* 1B isolates. In the present study, using a positive selection strategy, we identified three HPI-deleted mutants of *Y. enterocolitica* strain Ye8081. In these three independent clones, the chromosomal deletion was not limited to the HPI but encompassed a larger DNA fragment of approximately 140 kb. Loss of this fragment, which occurred at a frequency of approximately 5×10^{-7} , resulted in the disappearance of several phenotypic traits, such as growth in a minimal medium, hydrolysis of *o*-nitrophenyl- β -D-thiogalactopyranoside, Tween esterase activity, and motility, and in a decreased virulence for mice. However, no precise excision of the Ye8081 HPI was observed. To gain more insight into the molecular basis for this phenomenon, the putative machinery of HPI excision in *Y. enterocolitica* was analyzed and compared to that in *Y. pseudotuberculosis*. We show that the probable reasons for failure of precise excision of the HPI of *Y. enterocolitica* Ye8081 are (i) the interruption of the P4-like integrase gene located close to its right-hand boundary by a premature stop codon and (ii) lack of conservation of 17-bp *att*-like sequences at both extremities of the HPI. These mutations may represent a process of HPI stabilization in the species *Y. enterocolitica*.

Yersinia spp. are gram-negative bacteria belonging to the family *Enterobacteriaceae* (3). The genus is composed of 11 species which can be divided into three pathogenicity groups. Strains nonpathogenic for humans are found mainly in the environment and sometimes also as transient intestinal saprophytes. These include *Yersinia intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. mollaretii*, *Y. bercovieri*, *Y. aldovae*, *Y. rohdei*, *Y. ruckeri*, and *Y. enterocolitica* biotype 1A (38). Strains with a moderate level of pathogenicity are widespread in cold and temperate countries and are responsible for mild enteric infections in humans (38). They are not lethal for mice at low doses and correspond to *Y. enterocolitica* strains of biotypes 2 to 5 (14). The group of high-pathogenicity strains is composed of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* biotype 1B (46), which are all lethal for mice at low doses and cause disseminated infections in humans.

This high level of pathogenicity correlates with the presence of a pathogenicity island (PAI) called the high-pathogenicity island (HPI) in *Yersinia*, as it differentiates low- and high-pathogenicity strains (10). PAIs are large pieces of chromosomal DNA that carry virulence genes, possibly acquired by phage-mediated horizontal transfer (24). The HPI of *Y. enterocolitica* 1B (10) is a chromosomal fragment of 45 kb that carries virulence genes known as the yersiniabactin locus that are

involved in iron uptake (10, 39). Yersiniabactin is a siderophore (26) that endows the bacterium with the ability to acquire the iron molecules necessary for its in vivo growth and dissemination. Most of the length of the HPI is occupied by the yersiniabactin locus (Fig. 1), which comprises the genes *irp1* to *irp5*, involved in nonribosomal biosynthesis of the siderophore (22, 39), and the outer membrane siderophore receptor gene *fyuA* (27, 42). The G+C content of these genes is higher than the chromosomal background. The yersiniabactin locus is highly conserved among *Y. enterocolitica* 1B strains (10) and in the two other high-pathogenicity species, *Y. pseudotuberculosis* (7) and *Y. pestis* (2, 8, 20). The region located on the right side of this locus, between *irp2* and the right-hand boundary of the *Y. enterocolitica* HPI (Fig. 1), has not yet been sequenced. However, based on hybridization experiments with *Y. pestis* probes (8), it is most likely that this region carries genes recently described as composing another part of the yersiniabactin locus in *Y. pestis* (17). The right-hand border of the *Y. enterocolitica* HPI is defined by an *asn-tRNA* gene (10). In contrast, the \approx 15-kb region of the *Y. enterocolitica* HPI extending on the left side of the yersiniabactin locus (Fig. 1) is not well conserved among various isolates of this species (10) and is different from the regions found on the HPIs of the two other high-pathogenicity species (7, 8). The only genes identified until now on this 15-kb region correspond to a cluster of three repeated sequences (IS1400 [10], IS1328 [41], and RS3 [10]) (Fig. 1). The left-hand boundary of the island has not been precisely identified.

The HPI of *Y. enterocolitica* 1B thus shows nearly all of the criteria of a typical PAI as defined by Hacker and colleagues

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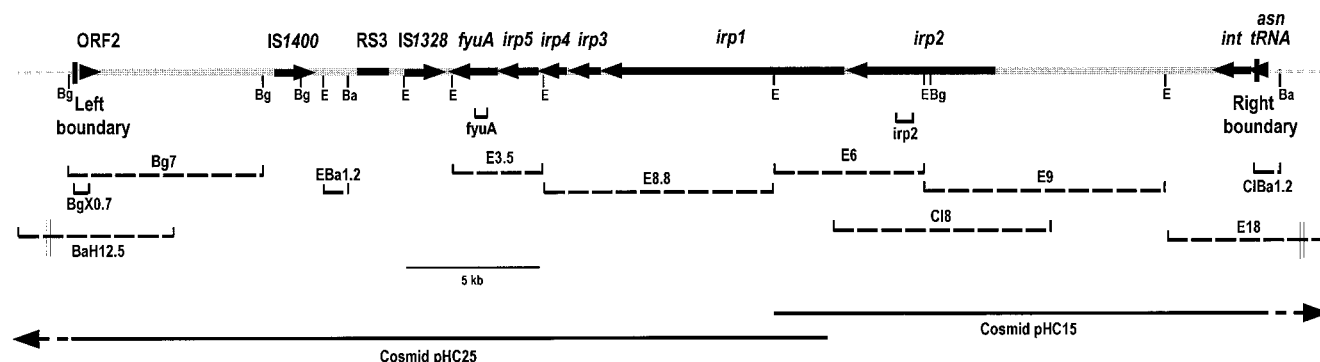


FIG. 1. Genetic organization of the HPI of *Y. enterocolitica* Ye8081. The HPI is represented by the gray line, and the identified genes are represented by arrows. Dashed lines below the diagram represent probes, named on the basis of their size in kilobases and on the restriction sites used to generate them. E, *EcoRI*; Bg, *BglII*; Ba, *BamHI*; X, *XhoI*; Cl, *ClaI*; H, *HindIII*.

(24): (i) it is a large chromosomal DNA fragment, (ii) it carries virulence genes essential for the expression of the high-virulence phenotype, (iii) it harbors several repeated sequences, (iv) it is bordered on one side by a *tRNA* gene, and (v) its G+C content is different from that of the rest of the chromosome. However, while PAIs are often described as mobile elements, and while the HPis of *Y. pestis* (18, 19) and *Y. pseudotuberculosis* (7) are able to undergo spontaneous deletion from the host chromosome, the *Y. enterocolitica* HPI seems to be stable. Our previous attempts to identify spontaneous HPI deletion mutants among natural isolates of *Y. enterocolitica* 1B or following repeated subcultures of high-pathogenicity strain Ye8081 were unsuccessful (16). To date, no precise excision of this island has been reported. The aim of this study was to confirm whether the HPI of *Y. enterocolitica* was a stable feature of this organism's genome and, if it was, determine the species-specific mechanism of stabilization.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Study of HPI stability was performed on strain Ye8081, a *Y. enterocolitica* biotype 1B serotype O:8 strain that was previously used to characterize the PAI of this species (10). Forty-one other strains of *Yersinia*, including 19 *Y. enterocolitica* strains of different bioserotypes, 11 *Y. pseudotuberculosis* strains of serotypes I to V, 3 *Y. pestis*, 2 *Y. bercovieri*, 1 *Y. frederiksenii*, 1 *Y. kristensenii*, 2 *Y. intermedia*, 2 *Y. mollaretii* strains, and 1 *Y. aldovae* strain were also studied. The *Escherichia coli* strains used were K-12 strain TG1 [$\Delta(lac-proAB)$ *supE* *thi* *hsdR5/F'* *traD36* *proA*⁺ *proB*⁺ *lacI*^q *lacZ* Δ M15] as host for M13 bacteriophage, TOP10F' [*F'*(*lacI*^q *Tn10* (Tet^r)) *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (Str^r) *endA1* *nupG*] for transformation with plasmid pCRII-TOPO (Invitrogen), and Epicurian Coli XL1-Blue [*recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* (F' *proAB* *lacI*^q Δ M15 *Tn10* (Tet^r))] (Stratagene) for electroporation of pBluescript and pUC19. *Yersinia* strains were grown at 28°C for 24 h in peptone broth (Difco) or in M9 minimal medium supplemented with 0.4% glycerol, 10 mM CaCl₂, 2 mM MgSO₄, and 1 μ g vitamin B₁ per liter or for 48 h on Trypticase soy agar (Diagnostics Pasteur) plates. *E. coli* strains were grown at 37°C for 24 h in Luria broth (Difco) or on Luria agar plates. When necessary, ampicillin at a concentration of 50 μ g/ml was added to the media. The plasmids used were pCRII-TOPO (Invitrogen), pUC19 (Biolabs), and pBluescript II KS⁺ (Stratagene).

DNA techniques. Isolation of genomic DNA was performed with an IsoQuick nucleic acid extraction kit (MicroProbe). Plasmid extraction was carried out according to the method of Birnboim and Doly (4). For Southern hybridizations, approximately 10- μ g aliquots of DNAs from the various *Yersinia* strains were digested with *EcoRI* and subjected to overnight electrophoresis at 50 V in 0.8% agarose gels. DNAs were transferred to nylon membranes (Hybond N⁺; Amersham) by using a vacuum blotting system (VacuGene XL; Pharmacia) and hybridized with the corresponding probes. Colony blot hybridizations were done on bacterial colonies spotted onto nylon filters and treated as previously described (35). Double-stranded DNA labeling was performed either radioactively with [α -³²P]ATP (NEN), using a Megaprime DNA labeling systems kit (Amersham), or nonradioactively with digoxigenin (DIG), either by incorporation of DIG-11-dUTP during PCRs or with the random primed labeling system (Boehringer

Mannheim). Plasmid or cosmid insert probes were purified by elution from an agarose gel with a QIAquick gel extraction kit (QIAGEN). Single-stranded oligonucleotides were labeled with DIG by using a DIG-oligonucleotide tailing kit (Boehringer Mannheim). The oligonucleotides used as probes or for probe amplification are listed in Table 1. Hybridizations were carried out at 65°C (DIG-labeled probes) or 68°C (radiolabeled probes) for random primed probes and at 54°C (BG1), 59°C (BG2), or 56°C (*att*-like) for DIG-labeled oligonucleotides.

PCR. PCRs were carried out in a 50- μ l reaction volume with an OmniGene thermal cycler (Hybaid). Fifty to 100 ng of target DNA, 200 μ M each deoxynucleoside triphosphate, 1 μ M each primer, and 1 U of *Taq* polymerase (Perkin-Elmer) or *Pfu* polymerase (Stratagene) were mixed in 1 \times polymerase buffer. Amplification involved 30 cycles, each consisting of a denaturation step of 30 s at 92°C, an annealing step of 1 min 30 s at 55°C, and a polymerization step of 1 min 30 s at 72°C, ending with a final cycle at 72°C for 10 min. Primers used for PCR (Table 1) were designed with the PC-Rare software (21).

Sequencing and sequence analysis. To sequence the *int* gene, a 1,872-bp fragment was amplified by PCR with primers int1 and int2 (Table 1), using the Ye8081 genomic DNA as template, and was subsequently cloned into the *EcoRI* and *BamHI* sites of plasmid pUC19. The insert was then ligated into the replicative form of bacteriophages M13mp18 and M13mp19 and sequenced by the dideoxy-chain termination method (47), using a Thermo Sequenase core sequencing kit with 7-deaza-dGTP from Vistra (Amersham) and a Vistra DNA Sequencer 725 (Amersham). A portion of the *int* genes of *Y. enterocolitica* strains IP845 and IP24764 was sequenced by amplification of a 336-bp fragment of their genomic DNAs by nested PCR with primers int1 and int2 for the first round and primers intC and intD for the second round (Table 1). The amplified fragments were then cloned into vector pCRII-TOPO (Invitrogen) and sequenced with a Cy5-AutoRead sequencing kit (Pharmacia), using the pgemD forward and M13 reverse primers and an annealing temperature of 65°C for 10 min, followed by incubation at 37°C for 10 min before cooling at room temperature for 10 min. An ALF express automatic sequencer (Pharmacia) was used for sequencing reactions. To sequence the left border of the Ye8081 HPI, the 0.7-kb *BglII*-*XhoI* fragment (BgX0.7 [Fig. 1]) that has previously been shown to correspond to the limit of the HPI (10) was cloned into the corresponding sites of pBluescript. Sequencing was performed by the dideoxy-chain termination method with Sequenase (United States Biochemical Corp.). Sequence analysis was performed with Assembly Lign, BLAST N, BLAST P (1), and Clustal V (28).

Pulsed-field gel electrophoresis. Genomic DNA was prepared in agarose plugs as previously described (23). After digestion with *SpeI*, *NotI*, *XbaI*, or *SrfI*, the macrorestriction fragments were resolved with a CHEF-DRIII apparatus (Bio-Rad Laboratories), using an electric field of 6 V/cm and an angle of 120°. Migration of the DNA fragments was performed in 0.5 \times Tris-borate-EDTA buffer and in 0.9% agarose gels at 17°C. Pulse times varied according to the size of the fragments to be resolved.

Pesticin production and selection of pesticin-resistant colonies. Pesticin was prepared according to the method of Toora *et al.* (51). Induction of pesticin production by *Y. pestis* EV76 was obtained by incubating the strains for 19 h in the presence of 0.75 μ g of mitomycin C (Sigma) per ml. *Y. enterocolitica* pesticin-resistant colonies were detected as previously described (7).

Bacterial motility testing. Motility of the parental strain *Y. enterocolitica* Ye8081 and of the three isogenic mutants Ye8081H-1, Ye8081H-2, and Ye8081H-3 was tested on tryptone swarm plates (1% Bacto Tryptone, 0.5% NaCl, 0.3% Bacto Agar). Approximately 5 \times 10⁵ bacteria were spotted onto these plates and incubated at 25 or 37°C for 24 h. Staining of flagella was performed as described elsewhere (45).

Biochemical tests. Phenotypic characterization of *Yersinia* strains was performed with API 20E and API 50CH strips (bioMérieux, Marcy l'Etoile, France).

TABLE 1. Primers used for PCR amplification or sequencing and oligonucleotides used as probes

Primer or oligonucleotide	Sequence	Size (bp) of amplified DNA		Template(s)	Amplified or target DNA
Primers					
BP3 forward	5'-TGCGCCATGCGGTCCATC-3'	712	Genomic DNA	Ye8081	<i>int</i>
BP4 reverse	5'-GGTGCATAAGATTCTCGG-3'				
int1 forward	5'-TTTGGATCCGATTTTACCCTACG-3' containing a <i>Bam</i> HI site	1,872	Genomic DNA	Ye8081, IP845, IP24764	<i>int</i>
int2 reverse	5'-TTTGAATTCTCAGCATCTGGGGTC-3' containing an <i>Eco</i> RI site				
intC forward	5'-GCGGGCTGCTGAACGTGGCTC-3'	336	Genomic DNA	Ye8081, IP845, IP24764	<i>int</i>
intD reverse	5'-GGAGGTGTGGTCACGCCGCCA-3'				
14 forward	5'-GCAGGCTATCGCCGCCTTGC-3'	884	Genomic DNA	Ye8081	<i>irp2</i>
18 reverse	5'-GCCGAAAGCCTGGCCTTTA-3'				
<i>fyuA</i> 1A forward	5'-GGCGGCGTGCCTTCTCGCA-3'	540	Genomic DNA	Ye8081	<i>fyuA</i>
<i>fyuA</i> 1B reverse	5'-GCTGCTTCCCGCGCCATAAC-3'				
<i>pgemD</i> forward	5'-Cyanine-d(GACGGCCAGTGATTGTAAT)		pCRII-TOPO		<i>int</i>
M13 reverse	5'-Cyanine-d(CAGGAAACAGCTATGAC)-3'				
YplA1	5'-GAGCCTCTGTGGCTCCCC-3'	1,830	Genomic DNA	Ye8081, Ye8081H-.1 to Ye8081H-.3	<i>ypl</i> locus
YplB2	5'-TTCAGTCACTTTGGATGT-3'				
Oligonucleotides					
12	5'-TTTGAGTCCAGTCAGA-3'				<i>att</i> -like site
BG1	5'-TGTGAAACTCTCAAAAATAACAGGTATAAT-3'				Left border region
BG2	5'-GCTTTTAGCATCTGCTCTTGTAAAGTAAGT-3'				Left border region

Hydrolysis of *o*-nitrophenyl- β -galactopyranoside (ONPG) was monitored by suspending bacteria grown on agar plates in a tube containing distilled water and an ONPG disc (Sanofi Diagnostics Pasteur, Paris, France) and incubating them for up to 24 h at 37°C in a water bath (33). Presence of pyrazinamidase was evaluated as previously described (29). DNase activity was determined by spotting the bacteria on DNase agar media (Diagnostics Pasteur) and incubating them at 28°C for 48 h (6). Presence of DNase was characterized by a translucent zone after washing with 1 N HCl solution. To test for Tween esterase activity, bacterial cells were spotted onto Tween 80 plates (containing, per liter, 10 g of pancreatic peptone [Prolabo], 5 g of NaCl, 0.1 g of CaCl₂, 4.63 ml of Tween 80 [Merck], and 12 g of Bacto Agar [Difco] [pH 7.4]). After incubation at 28°C for 24 h, a cloudy zone indicated the presence of the enzyme.

Animal infection and estimation of the LD₅₀. To measure the median lethal dose (LD₅₀) of the wild-type strain Ye8081 and of the Ye8081H-.1 mutant derivative, 0.5-ml aliquots of 10-fold serial dilutions of bacterial suspensions were inoculated intravenously into 16-day-old OF1 female mice (Iffa Credo, Lyon, France). Groups of five mice were used for each dilution, and five dilutions were used each time. In some experiments, mice were iron overloaded by intraperitoneal injection of 0.5 ml of saline containing 1 mg of ferric chloride prior to the bacterial challenge. LD₅₀s were calculated by the method of Reed and Muench (43).

Nucleotide sequence accession numbers. The sequences of the *int* gene and of the left border of the HPI have been deposited in the EMBL databank under accession no. AJ010281 and AJ238284, respectively.

RESULTS

Identification of deletion derivatives of *Y. enterocolitica* Ye8081. The absence of HPI deletion strains among natural isolates of *Y. enterocolitica* 1B (16) and our failure to obtain deletion of the island upon serial subcultures in vitro suggested that if the excision of the HPI does occur in *Y. enterocolitica*, it should be at a much lower frequency than in *Y. pseudotuberculosis* (7). Thus, in order to observe rare deletion mutants, we designed a positive selection procedure based on the fact that the yersiniabactin receptor FyuA which is located on the HPI is also the receptor for the bacteriocin pesticin (42). Pesticin acts like lysozyme in hydrolyzing murein lipoproteins on the

bacterial cell wall (17). Colonies deleted of the pesticin receptor should become resistant to the action of this molecule. Upon treatment of strain Ye8081 with crude extracts of pesticin, 800 colonies that grew in the presence of the bacteriocin were collected, spotted on nylon membranes, and hybridized with two PCR-amplified probes (*fyuA* and *irp2*) encompassing the HPI-borne *fyuA* or *irp2* gene (Fig. 1). Of the 800 pesticin-resistant colonies tested, 797 hybridized with both probes, indicating the presence of at least a portion of the HPI in these strains and suggesting that a mutation in the pesticin receptor may have been responsible for their resistance to the bacteriocin. The three remaining colonies, designated Ye8081H-.1, Ye8081H-.2, and Ye8081H-.3 and obtained from three independent platings, failed to hybridize with either the *fyuA* or the *irp2* probe. Southern hybridization of the *Eco*RI-digested DNA of the three mutants and of the parental strain Ye8081 with the Cl8 and E3.5 probes (Fig. 1) derived from cosmid clones pHC25 and pHC15 (10) confirmed the absence of these two DNA regions in the three mutant strains. Based on the estimated number of bacteria per bacteriocin-induced lysis plaque, the frequency of deleted clones of strain Ye8081 was estimated to be 5×10^{-7} .

To determine the extent of the deletion that encompasses the *fyuA* and *irp2* genes, eight cosmid-derived probes (BgX0.7, Bg7, EBa1.2, E3.5, E8.8, E6, E9, and ClBa1.2 [Fig. 1]) covering the HPI with the exclusion of the repeated elements (IS1400, RS3, and IS1328), were used to hybridize with the *Eco*RI-digested DNA of strain Ye8081 and of the three mutants. None of these eight probes hybridized with Ye8081H-.1, Ye8081H-.2, or Ye8081H-.3 DNA. Moreover, the two probes located at each extremity of the HPI (HB12.5 and E18 [Fig. 1]) and encompassing the flanking chromosomal regions did not hybridize with the DNAs of the mutant strains, indicating that

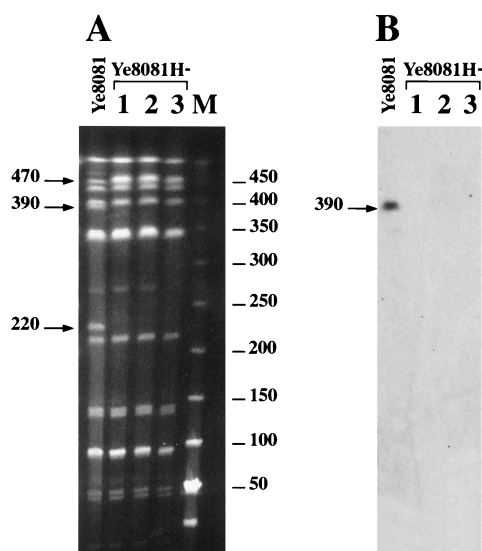


FIG. 2. Comparison of genomic restriction profiles of the parental strain Ye8081 and of the three deletion derivatives (Ye8081H-). (A) Pulsed-field gel electrophoresis of *SrfI*-digested genomic DNA. M, molecular weight markers, whose sizes (in kilobases; values have been rounded up) are indicated on the right. The arrows point to fragments that differed between the wild-type strain and the three deletion clones. (B) Southern hybridization of the *SrfI*-digested DNA of the four strains with either the BaH12.5 or the E18 probe. The arrow indicates the size of the hybridizing fragment.

the unstable regions do not correspond precisely to the HPI but extend beyond the island on both sides and cover a region at least 67 kb long.

Analysis of the genomic profiles of strain Ye8081 by pulsed-field gel electrophoresis after digestion with *SpeI*, *NotI*, *XbaI*, or *SrfI* showed that several restriction fragments differed between the parental strain and the three mutants. In contrast, the genomic profiles of these three independent deletion mutants were identical, whatever the enzyme used (data not shown), suggesting that the same chromosomal fragment was removed in the three clones. Moreover, with the *SrfI* enzyme, which gives a small number of restriction fragments, it was possible to show that the genomic profile of the wild-type strain Ye8081 differed from that of the three isogenic deletion derivatives by the presence of two fragments of approximately 220 and 390 kb and by the absence of a ≈ 470 -kb fragment (Fig. 2A). Both the HB12.5 and E18 probes located at each extremity of the island hybridized with the 390-kb *SrfI* fragment in strain Ye8081 but with no fragment in the deletion derivatives (Fig. 2B). By subtracting the sizes of these fragments, it was estimated that the region deleted in Ye8081H-1, Ye8081H-2, and Ye8081H-3 encompassed approximately 140 kb of chromosomal DNA.

Deletion of the 140-kb unstable fragment is accompanied by the loss of several phenotypic traits. Deletion of the 140-kb unstable fragment resulted in complete removal of the HPI and therefore the inability of the mutants to synthesize yersiniabactin (2, 39). Analysis of the impact of the HPI deletion on other phenotypic and metabolic properties showed that the three deletion derivatives differed from the parental strain Ye8081 by the alteration of four characters. First, while the parental strain and the three mutants grew equally well in a rich medium (peptone broth), the deletion derivatives exhibited defective growth in a minimal medium (M9). Second, the ability to hydrolyze ONPG was abolished in the three mutants. In the genus *Yersinia*, hydrolysis of ONPG is not due to a

β -galactosidase *sensu stricto* but to an ONPG-hydrolase (38). Third, all three deletion mutants were impaired in the ability to hydrolyze Tween 80. This Tween esterase activity is due to a phospholipase A enzyme whose genes (*ylpA* and *ylpB*) have been recently identified in strain Ye8081 (48). To determine whether this locus was present in the deletion clones, primers YplA1 and YplB2, located at the 5' extremity of *ylpA* and at the 3' end of *ylpB*, respectively, were used to amplify the entire locus by PCR. Fragments of the same size were obtained in the parental strain and the three mutants, indicating that the deletion did not remove this locus and suggesting that other elements necessary for the expression of a Tween esterase activity were altered. The fourth character lost by the mutants was motility. The genus *Yersinia* is characterized by the ability of the bacteria to be motile at 28°C but not at 37°C (except *Y. pestis*, which is always nonmotile) (38). As expected, both the wild-type strain and the mutant derivatives were motionless on swarm plates incubated at 37°C, but only the wild-type strain gained motility upon incubation at 25°C (Fig. 3A). Flagellum staining of 25°C-grown bacteria demonstrated that the deletion strains had lost the capacity to produce these motility elements (Fig. 3B).

Therefore, our data demonstrate that the 140-kb unstable fragment carries, in addition to the HPI, genes involved directly or indirectly in growth in a minimal medium, in ONPG and Tween 80 hydrolysis, and in expression of flagella at the surface of the bacteria.

Impact of deletion of the 140-kb unstable fragment on the virulence of the mutant strain. To evaluate the effect of the 140-kb chromosomal deletion on the virulence of *Y. enterocolitica*, the LD₅₀s of Ye8081 and Ye8081H-1 were estimated after intravenous injection into mice. The presence of the virulence plasmid pYV was checked prior to animal infection. The LD₅₀ of the mutant strain ($>10^6$ CFU) was more than 7,000 times greater than that of the parental strain (1.4×10^2 CFU). The decreased virulence of the mutant strain can be at least partly attributed to the deletion of the HPI, which is known to be important for *Yersinia* pathogenicity (2, 11, 42). A decrease in virulence was also observed when the *Y. enterocolitica* yersiniabactin receptor gene *fyuA* was mutagenized (42). However, the participation of the adjacent unstable chromosomal regions in this process could not be ruled out. High-pathogenicity *Y. enterocolitica* strains of biotype 1B are inherently lethal for laboratory animals, while low-pathogenicity strains of biotypes 2 to 5 are naturally devoid of the HPI and do not kill mice at low doses. Robins-Browne and Kaya Prpic (46) demonstrated that injection of the animals with iron and/or an exogenous siderophore (Desferal) prior to the bacterial challenge did not significantly change the virulence of the high-pathogenicity strains but increased the virulence of the strains of biotypes 2 to 5, by providing them with the iron molecules necessary for their *in vivo* growth and dissemination. To determine whether the decreased virulence of the mutant strains was essentially due to the loss of the iron-chelating system carried by the HPI, or whether the adjacent unstable regions could also be involved, mice were treated with ferric chloride before bacterial infection. The virulence of the high-pathogenicity strain Ye8081 (LD₅₀ of 3.1×10^2 CFU) was not modified by the iron treatment. In contrast, the pathogenicity of the mutant strain Ye8081H-1 increased (to an LD₅₀ of 2×10^5 CFU) upon iron treatment but remained significantly lower than that of the parental strain. Although the detrimental effect of iron on the mouse immune response may have contributed to the decreased LD₅₀, these results suggest that the reduced virulence observed upon deletion of the 140-kb chromosomal region is partly attributable to the loss of the

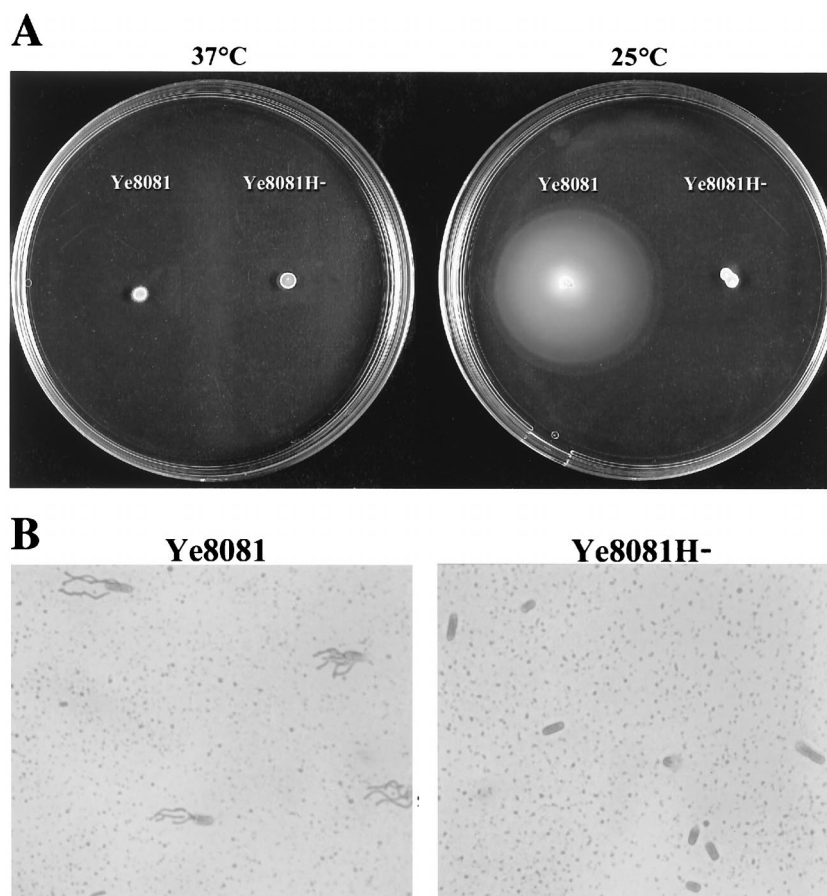


FIG. 3. Motility of the parental strain Ye8081 and of the deletion derivatives (Ye8081H-). (A) Swarm plates inoculated with the parental strain and one isogenic deletion mutant and incubated at 37 or 25°C. (B) Flagellum staining of the parental strain and of the isogenic mutant upon incubation at 25°C.

HPI but that other genes located on this unstable region also play a role in the virulence of strain Ye8081. It has recently been shown that mutagenesis of the phospholipase A locus of strain Ye8081 alters the virulence of the mutant strains for mice (48). It is thus likely that the abolition of the Tween 80 activity observed in our deletion mutants may have contributed to their reduced virulence.

Altogether our data demonstrate that a spontaneous 140-kb deletion which carries the HPI and genes involved in ONPG hydrolysis, Tween 80 esterase activity, motility, and pathogenicity can occur in the chromosome of strain Ye8081. However, our data also indicate that, in contrast to the HPI of *Y. pseudotuberculosis* (7), the homologous island of *Y. enterocolitica* 1B does not undergo precise excision.

The P4-like integrase gene is mutated in *Y. enterocolitica* 1B strains. The HPI of *Y. pseudotuberculosis*, which is capable of precise excision from the host chromosome (7), contains a gene homologous to the bacteriophage P4 integrase gene (*int*) (7, 25). An integrase gene almost identical to that of *Y. pseudotuberculosis* was identified at the same position on the HPI of *Y. pestis* (9). To determine whether the HPI of *Y. enterocolitica* 1B carries a similar *int* gene, the segment of the HPI located immediately downstream of the *asn-tRNA* locus of strain Ye8081 (Fig. 1) was amplified by PCR, cloned into pUC19 and bacteriophage M13, and sequenced. A 1,260-bp-long sequence homologous to the bacteriophage P4 integrase (40) gene (57.5% identity over 1,260 bp) and highly similar to the *int* gene of *Y. pseudotuberculosis* (98.8% identity over 1,260 bp)

was identified. The G+C content of this sequence was 52.9% and therefore, as is the case for other HPI-borne genes, much higher than the average G+C content of the host chromosome (46 to 48%). However, while the *Y. pseudotuberculosis int* sequence had the capacity to code for a 420-amino-acid (aa) protein (7), the Ye8081 sequence could encode only a 138-aa-long peptide because a T residue instead of a G at position 415 of the nucleotide sequence introduced a premature stop codon. To ensure that this stop codon was not an artifact generated during PCR amplification and to determine whether a truncated *int* gene was characteristic of all *Y. enterocolitica* 1B strains, the integrase region surrounding the premature stop codon was amplified and sequenced for two other *Y. enterocolitica* strains of biotype 1B: IP845 (serotype O:20) and IP24764 (serotype O:13 [20]). For this purpose, nested PCR with primer pairs *int1-int2* and *intC-intD* followed by cloning into pCRII-TOPO was carried out. The 336-bp amplified sequences of the two strains were identical to the Ye8081 sequence and exhibited the same stop codon interrupting the open reading frame (ORF). These results suggest that the *int* gene is commonly mutated in *Y. enterocolitica* 1B strains. Without this premature stop codon, the *Y. enterocolitica int* gene would have had the potential to code for a 420-aa integrase protein with 98.6% identity and 99.3% similarity to its *Y. pseudotuberculosis* homologue and with 49.5% identity and 77.6% similarity to the bacteriophage P4 integrase.

To analyze the distribution of the *int* gene within the genus *Yersinia*, a 712-bp probe generated by PCR with primers BP3

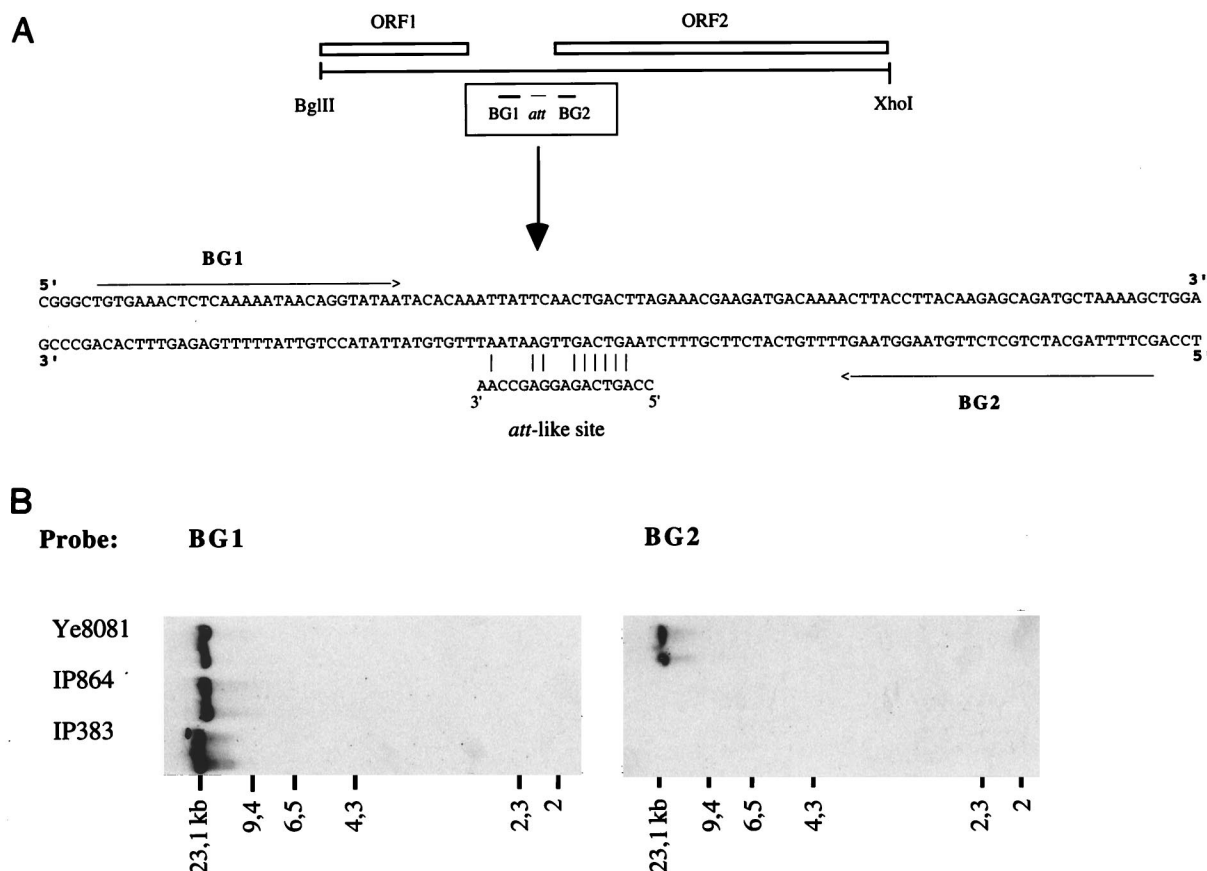


FIG. 4. (A) Schematic representation of the 707-bp *Bgl*II-*Xho*I region encompassing the left-hand boundary of the Ye8081 HPI and nucleotide sequence of the region where the degenerate *att*-like site was identified. (B) Hybridization of the *Eco*RI-digested genomic DNA of the three strains listed with BG1 and BG2 probes.

and BP4 (Table 1) was used to hybridize with the *Eco*RI-digested DNAs of various pathogenic and nonpathogenic species of *Yersinia* (see Materials and Methods). A perfect correlation was observed between the presence of the HPI and the *int* gene; i.e., the *int* gene was found in high-pathogenicity strains only (data not shown). Furthermore, under the stringent hybridization conditions used in our study, only one fragment hybridized with the probe, indicating that a single copy of the P4-like integrase gene is present on the chromosome of high-pathogenicity strains.

Our results thus demonstrate that although a well-conserved integrase gene is present on the HPI of *Y. enterocolitica* 1B, this gene is interrupted and therefore probably nonfunctional in this group of bacteria.

The 17-bp repeat located at the left-hand border of the HPI of *Y. enterocolitica* Ye8081 is degenerate. In *Y. pseudotuberculosis* (7, 25) and in *Y. pestis* (9), a 17-bp-long sequence resembling the *attP* site of bacteriophage P4 was identified in the same orientation at the two extremities of the island. Excision of the *Y. pseudotuberculosis* HPI probably occurs by recombination between the two copies of the 17-bp *att*-like repeats and results in the generation of a fusion fragment containing the 17-bp sequence at the junction site (7). Similar *att*-like repeats at the borders of the Ye8081 HPI were sought. Analysis of the previously published sequence of the *asn-tRNA* locus bordering the right-hand extremity of the HPI of strain Ye8081 (10) revealed the presence of a 17-bp sequence (5'-CCAGTCAG AGGAGCCAA-3') identical to the HPI flanking sequence in

Y. pseudotuberculosis and *Y. pestis*. To determine whether this sequence was also present at the other extremity of the Ye8081 HPI, the DNAs of cosmid clones pHC25 and pHC15 (Fig. 1) were subjected to single and/or double digestion with *Eco*RI, *Bam*HI, *Bgl*II, and *Cla*I and hybridized with a 12-nucleotide (nt)-long oligonucleotide (Table 1) covering a portion of the *att*-like site. In cosmid pHC15, one hybridizing fragment having the expected size and corresponding to the cosmid segment carrying the *asn-tRNA* gene was recognized for each digestion (data not shown). In contrast, no restriction fragment from cosmid pHC25 hybridized with the probe, indicating that the portion of the 17-bp *att*-like site present on the probe was either absent or degenerate at the left-hand extremity of the HPI.

To further investigate this point, the 0.7-kb *Bgl*II-*Xho*I region previously defined by hybridization as containing the left-hand border of the HPI (10) was cloned into plasmid pBlue-script and sequenced. Two partial ORFs designated ORF1 and ORF2 were identified (Fig. 4). The predicted 66-aa product of ORF1 was similar (35% identity and 59% similarity over 62 aa) to the C-terminal part of the fructose-specific IIA/FPR component of the phosphoenolpyruvate-dependent sugar phosphotransferase system of *Haemophilus influenzae* (44) and of other bacterial species such as *Salmonella typhimurium* and *E. coli*. ORF2 had the capacity to encode the first 121 aa of a protein highly similar (87.6% identity and 97.5% similarity over the entire 121 aa) to the N-terminal portion of a hypothetical 19.9-kDa protein of *E. coli*, whose gene is located in

the *mhpT-adhC* intergenic region (5). When the 0.7-kb *BglII-XhoI* fragment which contains these two partial ORFs was hybridized with the genomic DNAs of *Y. pestis* and *Y. pseudotuberculosis*, no fragment was recognized, indicating that this region is not conserved in the two other pathogenic species of *Yersinia*. A FASTA search of a nucleotide motif corresponding to the 17-bp *att*-like site on the 707-bp *BglII-XhoI* sequenced fragment identified a region located between ORF1 and ORF2 and containing a short stretch of 6 contiguous nt (5'-AGTCA G-3') identical to those of the *att*-like site and a total of 9 identical nt over the 17 nt (Fig. 4). This moderate level of identity may indicate either that this region of partial identity is not the border of the HPI or that this border contains a degenerate *att*-like site. To determine whether this region was indeed the left-hand boundary of the HPI, two oligonucleotides (BG1 and BG2) flanking the putative *att*-like site (Fig. 4) were hybridized with the *EcoRI*-digested genomic DNA of the high-pathogenicity strain Ye8081 and of the low-pathogenicity strains *Y. enterocolitica* IP864 (biotype 4 and serotype O:3) and IP383 (biotype 2 and serotype O:9). The BG1 probe hybridized with the DNA of both the high- and low-pathogenicity strains, while BG2 hybridized only with the DNA of the high-pathogenicity strain (Fig. 4). Since the HPI has been defined as the portion of DNA encompassing the yersiniabactin iron uptake machinery which is found uniquely in high-pathogenicity strains (10), it can be concluded from these results that the boundary of the *Y. enterocolitica* HPI is located between BG1 and BG2 and therefore that it most likely corresponds to the degenerate *att*-like site identified (as HPI excision is disabled in *Y. enterocolitica*, experimental evidence for this is not available). Our data also indicate that a protein highly similar to an as yet uncharacterized protein of *E. coli* is encoded by the left-hand portion of the HPI.

These results thus demonstrate that in contrast to the HPI of *Y. pseudotuberculosis* and *Y. pestis*, the HPI of *Y. enterocolitica* Ye8081 contains only one conserved 17-bp *att*-like site at its right-hand boundary and no identical sequence at its left-hand boundary.

DISCUSSION

A previous analysis of various *Yersinia* species demonstrated that the HPI-borne *irp2* gene was unique to high-pathogenicity strains (16). Within this group of bacteria, we showed that some isolates of *Y. pestis* and *Y. pseudotuberculosis* had lost this gene and had a reduced virulence in the mouse model. This was not the case for *Y. enterocolitica* 1B strains, for which no *irp2*-deleted natural isolates were observed (16). Equally, attempts to obtain *irp2*-deletion mutants of strain Ye8081 by repeated subcultures, UV irradiation, or treatment with mitomycin C in search of *irp2*-non-hybridizing clones were unsuccessful (9a). This led us to conclude that the HPI of *Y. enterocolitica* is more stable than the islands of the two other high-pathogenicity species. The possibility of a low-frequency HPI deletion in Ye8081 could nonetheless not be eliminated. Using another strain of *Y. enterocolitica* 1B (WA), Rakin and Heesemann (41) identified some colonies with deletions of both *fyuA* and *irp2* or of *fyuA* alone (but not *irp2* alone). The former mutants may have represented HPI deletions, but they were not characterized. To screen a high number of Ye8081 colonies for the loss of the HPI, we developed a positive selection strategy based on the dual function of FyuA as a receptor for both yersiniabactin and the bacteriocin pesticin (27, 42). Using this strategy, we were able to obtain three Ye8081 mutants lacking an approximately 140-kb fragment encompassing the whole of the HPI and adjacent chromosomal sequences. The

fact that the genomic profiles of the three independently obtained clones were identical suggests that they shared a common mechanism of deletion. Characterization of this mechanism was beyond the scope of this study but deserves further investigation.

Surprisingly, deletion of the HPI appears to result from different mechanisms in the three highly pathogenic *Yersinia* species. In *Y. pestis*, this deletion occurs at a frequency of 10^{-5} by homologous recombination between two *IS100* copies flanking a large 102-kb chromosomal region named the *pgm* locus (18, 19). In *Y. pseudotuberculosis*, the HPI excises precisely from the chromosome at a frequency of $\approx 10^{-4}$, probably by site-specific recombination (7). In *Y. enterocolitica* 1B, we found that the HPI is lost a frequency of $\approx 5 \times 10^{-7}$ by deletion of a 140-kb unstable chromosomal fragment. Despite these apparent differences, the three HPIs superficially appear to have the same genetic apparatus for site-specific recombination, i.e., a site-specific recombinase (the P4-like integrase) encoded within the excisable DNA downstream of the *asn-tRNA* locus and short flanking direct repeat sequences (the 17-bp P4 *att*-like sites), representing the target sites for the recombinase. However, while the *int* genes of *Y. pseudotuberculosis* (7, 25) and *Y. pestis* (9) are intact and presumably have the capacity to code for a potentially functional integrase protein, the *int* gene of *Y. enterocolitica* 1B is prematurely interrupted and one of the 17-bp flanking regions is incompletely conserved. Whether a functional integrase is required for PAI excision is not clear. In the case of uropathogenic *E. coli*, both PAI I and PAI II have the capacity to excise from the chromosome upon recombination between direct flanking repeats (6) despite the fact that the integrase genes present on both PAIs are cryptic (24). On the other hand, the mobile PAI of *Staphylococcus aureus* (34) and the symbiosis island of rhizobia (50) both carry an intact integrase gene adjacent to one boundary of the island, closely resembling the *Y. pseudotuberculosis* HPI, which is also mobile (7). Furthermore, it has been shown that increased expression of *slpA*, a gene closely related to P4 *int*, leads to the excision of the cryptic P4-like prophage in *E. coli* (31). The participation of the integrase gene in the PAI excision process thus appears likely but remains to be demonstrated.

Site-specific recombination also requires a target DNA sequence specifically recognized by the recombinase. In the PAIs of several bacterial species, short direct repeats, often homologous to phage attachment sites, are present at each extremity. This is the case for the PAIs of *Helicobacter pylori* (12), *H. influenzae* (36, 37), *Vibrio cholerae* (30, 32), *S. aureus* (34), *Dichelobacter nodosus* (13), and rhizobia (50) among others. The sizes of these repeats vary between 135 and 16 nt. Interestingly, PAIs that have retained the capacity to excise precisely from the chromosome have similar-size repeats at their boundaries: 17 nt for the PAI of *S. aureus*, 17 nt for the symbiosis island of rhizobia, 16 nt for PAI I of *E. coli*, 18 nt for PAI II of *E. coli*, and 17 nt for the HPI of *Y. pseudotuberculosis*. Therefore, the conservation and the size of these repeats may be crucial for precise excision of the intervening DNA. In the case of *Y. enterocolitica* 1B, the putative *att*-like site situated at the left-hand boundary is degenerate and shares only 6-nt perfect identity with the right-hand repeat. This region of perfect identity is probably too short to function efficiently as the target site for a recombinase.

The capacities of precise excision of the HPI of *Y. pseudotuberculosis* and *Y. pestis* may not differ drastically. In *Y. pseudotuberculosis*, the HPI can precisely excise at the *att*-like sites and possess a potentially functional *int* gene. In *Y. pestis*, two perfectly conserved 17-nt flanking *att*-like sites and an

intact P4-like integrase gene are also present on the HPI (9), suggesting that this island has retained the potential for precise excision. Absence of detection of *Y. pestis* strains deleted precisely of the 35-kb HPI could be attributable to a higher frequency of deletion of the entire 102-kb unstable *pgm* locus compared to the frequency of excision of the HPI alone. In contrast to *Y. pestis* and *Y. pseudotuberculosis*, the HPI of *Y. enterocolitica* 1B carries mutations in the excision apparatus that probably account for its inability to excise precisely from the chromosome.

Therefore, this study demonstrates that the *Y. enterocolitica* HPI is located on a 140-kb unstable chromosomal fragment that can delete at a low frequency. However, this HPI has lost the capacity to excise precisely from the chromosome, probably because both the excision protein and its target site are now defective. To our knowledge, the *Yersinia* HPI is the only PAI found in various species within the same genus, and in other bacterial genera such as *Klebsiella* (15), *Citrobacter* (15), and *E. coli* (49), suggesting horizontal dissemination. We hypothesize that the differences we observe between the HPI in different species of *Yersinia* represent early steps in the evolution of this PAI. Progressive mutations in the excision machinery of the HPI are the first prerequisite for permanent acquisition of the PAI-specific virulence factors by the host genome. These mutations may be advantageous for the bacteria by facilitating their dissemination into mammalian hosts, but they are disadvantageous for the horizontal spread of the element among different bacterial groups. They would be expected to occur long before progressive adjustment of the island's G+C content to that of the chromosome and the disappearance of the scars that signal its probable acquisition by a bacteriophage.

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